An active attachment biofilm model to develop anti-caries strategies

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Chapter 4

The Effects of Propidium Monoazide Treatment on the Measured Composition of Polymicrobial Biofilms after Treatment with Chlorhexidine.

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Abstract

Background
The treatment of polymicrobial biofilms with antimicrobial compounds results in not only an overall loss of viability but also compositional shifts. While DNA-based technologies may be more appropriate for the assessment of bacterial composition than culturing, these techniques amplify DNA from both live and dead cells. Propidium monoazide (PMA) has been used to discriminate between live and dead cells by blocking the DNA from membrane-damaged cells from being amplified.

Aim
This study evaluated the use of PMA in a saliva-derived polymicrobial biofilm model subjected to a single chlorhexidine treatment.

Materials and Methods
The effects of PMA on viable cells were tested using both untreated and PMA-treated saliva as an inoculum. Viability was determined by plate counts, metabolic activity was determined by lactic acid production, and biofilm composition was assessed with 16S rRNA gene amplicon sequencing.

Results
Exposure to a 0.2% chlorhexidine rinse (Meridol Perio) reduced the viability and metabolic activity of 48 h biofilms. The shift in biofilm composition observed after the chlorhexidine exposure was enhanced after a post-rinse PMA treatment. PMA treatment had a small effect on the measured composition of water-rinsed biofilms. Treating saliva with PMA reduced bacterial viability and shifted the bacterial composition of saliva and saliva-derived biofilms.

Conclusion
The removal of DNA from non-viable cells with PMA treatment was shown to elicit an improvement in the detection of shifts in in vitro polymicrobial biofilms after antimicrobial treatment. However, PMA also influenced the ability of cells to grow, indicating that PMA should be used with caution.
Introduction

Developing new preventive compounds against oral pathogens requires the use of realistic screening models. Bacteria in biofilms are more resistant to treatments with antimicrobials compared with their planktonic counterparts (Tenover, 2006). Therefore, evaluating new compounds requires the use of biofilms rather than liquid cultures. Moreover, polymicrobial interactions in multispecies biofilms contribute to resistance to antimicrobials and host immunity (Kara et al., 2006; Burmølle et al., 2006; Luppens et al., 2008; Ramsey et al., 2009). Recently, an active attachment biofilm model was developed to allow for the high-throughput screening of oral care products (Exterkate et al., 2010). This model involves saliva-derived polymicrobial biofilms. In addition to overall loss of viability as a result of antimicrobial treatment, shifts in biofilm composition need to be monitored. The composition of polymicrobial biofilms is difficult to determine using culturing techniques, therefore, the use of DNA-based methodologies is a more appropriate alternative.

Although DNA-based methodologies have advantages over culturing techniques (Sanz et al., 2004), they also have a major drawback. DNA-based technologies do not allow for the discrimination between live and dead cells. When evaluating antimicrobial treatments, this might lead to an underestimation of treatment effects, as the DNA of non-vital cells is included in the analysis.

Treatment with propidium monoazide (PMA) has been proposed to overcome this problem. Cells are incubated with PMA allowing the PMA to penetrate cells with compromised membranes and bind to their DNA. Photolysis of PMA using bright visible light produces a nitrene that can form a covalent link to DNA and other molecules. Subsequently, the DNA cannot be amplified by PCR. The unbound PMA that remains in solution, is simultaneously inactivated by reacting with water molecules. The resulting hydroxylamine is no longer capable of covalently binding to DNA (Nocker et al., 2006). PMA does not penetrate cells that have an intact membrane, and it would therefore be suitable to help discriminate between live and dead cells. This concept has been tested in various studies on single species (Nocker et al., 2006; Nocker et al., 2007), oral pathogens (Loozen et al., 2011) and complex samples (Nocker et al., 2009; Lin et al., 2011). Nevertheless, these studies have only
produced limited data on the necessity of PMA treatment when compositional shifts are determined after mild antimicrobial treatments.

The aim of this study was to evaluate the use of PMA to discriminate between live and dead cells in a saliva-derived polymicrobial biofilm model after antimicrobial treatment. Moreover, the hypothesis that PMA does not affect viable cells was tested by treating saliva with PMA prior to biofilm formation and comparing the outcome to biofilms derived from untreated saliva. Inactivated PMA was used as a control to test for the possible effect of PMA on cell growth.

![Figure 1](image)

*Figure 1*

*Experimental design resulting in 12 groups of 4 biofilms each (grey boxes).*
Materials & Methods

Experimental design

The experimental design is shown in Figure 1. Saliva was collected on ice from a single donor, thoroughly mixed and split into portions. Saliva was left untreated, treated with PMA or treated with inactivated PMA. Subsequently, the saliva was diluted 2-fold with 60% sterile glycerol to protect the bacterial cells from cryodamage. Saliva was stored at -80°C until use. All salivary samples were processed in parallel to avoid differences in exposure to air and processing time.

Each of these saliva samples was used to inoculate a biofilm model. Biofilms were grown for 48 h and then treated with either water or 0.2% chlorhexidine. After harvesting the biofilms, the dispersed biofilms were either left untreated or treated with PMA. This design resulted in 12 experimental groups with 4 biofilms each.

Biofilm model

The biofilm model (Exterkate et al., 2010) consisted of a custom-made stainless steel lid with 24 clamps that contained glass cover slips (Ø 12 mm, Menzel, Braunschweig, Germany) used as substrata to grow biofilms. After assembling the lid with the substrata, the model was autoclaved. The lid fits onto standard polystyrene 24-well plates (multiwell plates, Greiner Bio-One, Alphen a/d Rijn, The Netherlands). The experimental design required two models. The models were operated in parallel.

Propidium monoazide treatment

A total of 1 mg propidium monoazide (Biotum Inc., Hayward, USA) was dissolved in 100 µl 20% DMSO. Approximately 2.5 µl of PMA was added to 500 µl saliva or suspended biofilm, incubated in the dark for 5 min and then exposed to intense light for 2 min using a 650 W halogen lamp placed 25 cm from the samples. The samples were kept on ice during this procedure.

Stock PMA was inactivated by exposing the 1 mg/100 µl stock PMA solution to intense light for 2 min (Nocker et al., 2006). Treatment with inactivated PMA followed the procedure described above.
Biofilm formation

The model was inoculated with 50-fold-diluted saliva in semi-defined medium consisting of 2.5 g/l mucin (Sigma M-2378), 2.0 g/l bactopeptone (Difco 0118-01-8), 2.0 g/l trypticase peptone (BBL 211921), 1.0 g/l yeast extract (Bacto 212750), 0.35 g/l NaCl, 0.2 g/l KCl, 0.2 g/l CaCl₂, 0.001 g/l hemin (Sigma H-1652), 0.0002 g/l vitamin K₁ (Mc Bain, 2005), 0.2% sucrose and 50 mmol/L PIPES at pH 7.0.

Biofilms were produced by adding 1.5 ml of the inoculation medium to each well. The model was subsequently incubated anaerobically (10% CO₂, 10% H₂ and 80% N₂) for 8 h at 37 °C. After this initial inoculation period, the lid was transferred to a new plate containing fresh medium (without bacteria). Medium was refreshed after 8, 24 and 32 h, with a total biofilm growth period of 48 h.

Treatment with 0.2% chlorhexidine (active rinse)

After 48 h, the biofilms were treated with either water or 0.2% chlorhexidine (meridol® perio Chlorhexidine 0.2%, GABA International AG, Switzerland). This treatment procedure has been described in detail previously (Exterkate et al., 2010). Briefly, the lid was transferred to a new plate containing 1.5 ml/well of the treatment solution and incubated for 5 min at room temperature. After the treatment, the biofilms were rinsed three times with cysteine peptone water (CPW). Treatment with 0.2% CHX or water will be referred to as rinses to avoid confusion with PMA treatment.

Acid production assay

At the end of the biofilm formation period and after the subsequent treatment, the lid was placed on a new plate containing 1.5 ml/well BPW (buffered peptone water) with 0.2% sucrose. The model was incubated anaerobically for 3 h at 37 °C. The amount of lactic acid formed during this period was analysed using a colorimetric assay (van Loveren et al., 2006).
**Determination of CFU**

After treatment with PMA or inactivated PMA, the saliva was diluted and plated in duplicate on tryptic soy agar blood (TSAB) plates to obtain total counts. The plates were incubated for 48 h at 37°C under anaerobic conditions (10% CO₂, 10% H₂ and 80% N₂).

The glass discs with the biofilms were removed from the lid and transferred into 2 ml PBS (phosphate-buffered saline). The biofilms were dispersed using a sonicator, and a single series of dilutions was made and plated on TSAB plates to obtain total counts. Samples for CFU counts were taken before the post-rinse PMA treatment.

**Post-rinse treatment of harvested biofilms with PMA**

After dispersing the biofilms in 2 ml PBS, aliquots of 1400 µl were centrifuged and resuspended in 500 µl of TE buffer. The samples were left untreated or treated with PMA.

**DNA extraction, amplicon preparation and pyrosequencing**

Salivary DNA was extracted as described previously (Crielaard et al., 2011), quantified (Quant-iT™ PicoGreen® dsDNA Reagent and Kits, Molecular Probes Inc, Willow Creek) and stored at -20°C until further analysis.

Barcoded amplicon libraries of the small subunit ribosomal RNA gene V5-V7 hypervariable region were generated for each of the samples as described previously (Kraneveld et al., 2012), pooled and sequenced using the Genome Sequencer FLX Titanium system (Roche, Basel, Switzerland).

**Sequencing data analysis**

The sequencing data were processed using QIIME (Quantitative Insights Into Microbial Ecology) version 1.4.0 (Caporaso et al., 2010), as described previously (Kraneveld et al., 2012). To allow for comparisons among different samples, the dataset was normalised to 2800 reads/sample. Then, phylogenetic measures of community β diversity, including unweighted UniFrac, a qualitative measure, and weighted UniFrac, a quantitative measure (Lozupone et al., 2007), were applied.
Principal coordinate analysis (PCoA) was used to compare groups of samples based on weighted UniFrac distance metrics. Next, to phylogenetic measures of community β diversity (UniFrac distance data), an OTU-based β diversity approach was used. For this, a normalised OTU abundance table was used in principal component analysis (PCA) using PAST software (Hammer et al., 2001).

**Statistical analyses**

Data were analysed statistically using anova with post-hoc Tukey’s test (IBM SPSS version 20).

**Results**

*CFU counts and lactic acid production.*

The water-treated 48 h biofilms formed with the three types of salivary inocula showed comparable CFU counts (fig. 2A). Rinsing with 0.2% CHX resulted in a decrease in CFU counts. This decrease was larger for biofilms that were formed from PMA-treated (active or inactivated) saliva. Biofilms formed from PMA-treated (active or inactivated) saliva showed a larger capability to form lactic acid, but rinsing with 0.2% CHX resulted in a strong reduction of acid production for all types of biofilms (fig. 2B).
Figure 2. Panel A. Total CFU of biofilms (average ± s.d.) formed from differently pre-treated saliva after a water or chlorhexidine rinse (N=12). Bars with different characters were significantly different.
Panel B. Lactic acid production of biofilms (average ± s.d.) formed from differently pre-treated saliva after a water or chlorhexidine rinse (N=12). Bars with different characters were significantly different.
Sequencing data analysis

Effects of pretreatment of saliva inocula with PMA

When saliva was treated with PMA or inactivated PMA, the CFU count decreased by 76% and 71%, respectively. The composition of the saliva also changed as a result of PMA treatment (fig. 3). The total number of OTUs decreased from 86 in untreated saliva to 68 in PMA-treated saliva. Treating saliva with inactivated PMA did not reduce the number of OTUs (85), but it did change the composition. The genera *Neisseria* and *Haemophilus* were affected to a large extent, as their relative abundance was decreased as a result of treatment with either PMA or inactivated PMA.

![Figure 3. The composition of differently pre-treated saliva that was used as inocula for biofilm formation. Data show bacterial taxa (family or genus if only one genus comprised the family or unclassifiable bacteria within one phylum). A version in colour is available in Appendix 1, page 122.](image)

Biofilms that were formed with the different inocula were dominated by genera *Streptococcus* and *Veillonella* (fig. 4). Pre-treating saliva with PMA (active or inactivated) resulted in biofilms that contained fewer OTUs (19 (SD 4) for the PMA pre-treated saliva vs 34 (SD 4) for the untreated saliva).
Effects of 0.2% chlorhexidine on biofilms

Rinsing with 0.2% CHX resulted in a decrease in the proportion of streptococci irrespective of the inoculum (fig. 4). When all data from the different inocula were combined the proportion of streptococci showed a statistically significant decrease from 58% (SD 12) to 45% (SD 8) after rinsing with 0.2% CHX, but without a post-rinse PMA treatment (fig. 4, the bars indicated with post rinse treatment “control”). Principal component analysis (PCA) revealed that the CHX rinse had an effect on sample clustering in the absence of post-rinse PMA treatment (fig. 5, black squares vs grey squares). These results were independent of the inoculum used. It should be noted that component 1 explained 95% of the variation, and component 2 only explained 3% of the variation, indicating that biofilms differ mainly on the x-axis of the graph.
Principal coordinate analysis (PCoA) of weighted UniFrac distances revealed that biofilms rinsed with 0.2% CHX but without post-rinse PMA treatment overlapped with those of the water rinse groups (fig. 6, black squares vs grey squares).

**Effects of post-rinse PMA treatment**

The post-rinse treatment with PMA enhanced the observed compositional shifts (fig. 4), for the combined data of all inocula the proportion of streptococci decreased statistically significant from 45% (SD 8) without PMA treatment to 20% (SD 7) with PMA treatment (fig. 4, the bars indicated with rinse “0.2% CHX”). The effect of post-rinse treatment with PMA on water-rinsed biofilms was smaller and not statistically significant (58% (SD 12) without PMA treatment to 65% (SD 9) with PMA treatment. (The bars indicated with rinse “water”).
Principal component analysis (PCA) revealed a minor effect of post-rinse treatment with PMA on water-rinsed biofilms (fig. 4) (grey squares vs grey circles). A post rinse PMA treatment on the 0.2% CHX rinsed biofilms caused the PMA treated biofilms (black circles) to cluster away from the non-PMA treated biofilms (black squares). The observed differences for the 0.2% CHX rinse (black squares vs grey squares) were enhanced by the post-rinse PMA treatment (black circles vs grey circles).

Figure 6. Principal coordinate analysis plot of the first two coordinates of the weighted UniFrac distance data from biofilms formed from differently pre-treated saliva (indicated by the data point labels) after a water rinse (grey symbols) or chlorhexidine rinse (black symbols) and a post-rinse treatment with PMA (circles) or control (untreated, squares).

Principal coordinate analysis showed a minor effect of post-rinse PMA treatment on water rinsed biofilms (fig. 6, grey circles vs grey squares). A post-rinse PMA treatment on 0.2% CHX rinsed biofilms caused the PMA-treated biofilms (black circles) to separate from the untreated biofilms (black squares). Post-rinse PMA treatment also caused the 0.2% CHX rinsed biofilms (black circles) to cluster away from the water-rinsed controls (grey circles), suggesting quantitative changes in biofilm composition.
Discussion

Our data show that compositional shifts in biofilms after a single exposure to 0.2% CHX, as determined by 16S rRNA gene amplicon sequencing, are partly obscured by the presence of DNA from non-viable cells. Introducing a PMA treatment step increased the compositional differences between water-rinsed and 0.2% CHX-rinsed biofilms. The composition of water-rinsed biofilms did not change substantially as a result of post-rinse treatment with PMA. Both, the biofilm viability, as determined by CFU counts, and metabolic activity decreased as a result of a single 0.2% CHX treatment.

The finding that PMA treatment enables the distinction between live and dead cells in PCR is not new. A number of authors have addressed this issue, and the method has been used in various applications. Loozen et al. (2011) showed that this approach could be used to enumerate dental pathogens. Our paper is the first to report the necessity of PMA treatment in evaluating the efficacy of a mild antimicrobial treatment of biofilms. The susceptibility of individual species to antimicrobial treatment may vary, which might lead to compositional shifts, in addition to the observed overall killing effect. This finding was observed in the current study.

Whether PMA only blocks DNA from cells with compromised membranes has been extensively studied without convincingly showing that PMA is 100% selective. In most studies, controls with 100% live cells still showed an effect of PMA treatment (Loozen et al., 2011, Nocker et al., 2009). This finding could be due to the presence of a small proportion of dead cells in the 100% live cell control, but the possibility that PMA could – to a limited extent – enter viable cells cannot be ruled out. In the current study, we aimed to prove the selectivity of PMA by pre-treating saliva with PMA and using the pre-treated saliva to form biofilms. The data showed that PMA inhibited the growth of cells, resulting in lower CFU counts for pre-treated saliva. In addition to blocking cell growth, a shift in the composition of the saliva as a result of PMA treatment was observed. However, this shift was a combination of the presence of non-viable cells in saliva and a possible effect of PMA treatment on viable cells.
The biofilms formed with the pre-treated saliva showed a reduction in the number of OTUs, indicating that PMA treatment influenced the growth ability of a variety of cells.

The inhibition of cell growth by PMA treatment has not been reported previously. PMA is generally used just prior to DNA extraction and amplification. However, from the current data, it appears that PMA can interact with cells that still have the capability to grow.

Light-inactivated PMA was used as a control. Nocker et al. (2006) and others have described that an excess amount of PMA reacts with water during exposure to intense light, thereby deactivating PMA. Apparently, this approach failed for the PMA stock solution, as inactivated PMA still influenced the bacterial composition of saliva to a large extent. However, inactivation was successful in the pre-treatment of saliva and the post-treatment assay, otherwise, the excess amount of PMA would not have been deactivated and would have inhibited DNA amplification.

The pre-treatment of saliva with (inactivated) PMA resulted in three different inocula, which produced biofilms that were somewhat different. PMA treatment did not affect species that were predominantly present in the 48 h biofilms (Veillonella and Streptococci). But the pre-treated saliva’s did produce biofilms that had lower CFU counts, higher lactic acid production and were more susceptible to the CHX rinse. Although the differences were statistically significant they were small. The PMA treatment of water-rinsed biofilms also resulted in a compositional shift. This shift most likely primarily reflects the presence of dead cells in the biofilms as a result of the 48 h growth period, although it cannot be excluded that PMA might also block the DNA of viable cells to some extent. The latter problem has been investigated by Fittipaldi (2011) in an elegant design, and the authors reported that only a small percentage of the control population should be regarded as false positive, i.e. found to be non-viable using PMA treatment but actually viable. Nevertheless, the compositional shift in the 0.2% CHX-treated biofilms after PMA treatment was far larger than in the water-rinsed biofilms, indicating that PMA treatment enhanced the observed compositional shift.
As any other molecular-biology-based method, 16S rRNA gene amplicon sequencing suffers from typical bias of DNA extraction and amplification, such as selectivity of primers and intrinsic differences in the amplification efficiency of templates (Zaura, 2012). Therefore studies based on these methods require scientifically sound experimental design with replicate samples (Rogers and Bruce, 2010). We have analyzed four replicate biofilms per group, allowing statistical analyses of the data. To reduce potential amplification and sequencing errors in the data, we have applied stringent data quality filtering protocol, including data denoising and removal of chimeric sequences.

The experiment was performed with saliva from a single donor. The main focus was on the effect that a PMA treatment has on the measured composition of the water- or CHX rinsed biofilms. The variation between donors in both the effect of the CHX rinse or the effect of PMA treatment was not the aim of the current study.

We conclude that a mild anti-microbial treatment resulted in shifts in the composition of polymicrobial biofilms, but these shifts were partly obscured by the presence of DNA from non-viable cells. The removal of DNA from non-viable cells with PMA treatment appears to be an important improvement in the detection of shifts in in vitro polymicrobial biofilms.

The finding that PMA influences the viability of cells is an issue that needs to be investigated further. Whether non-viable cells affect the reported composition of in vivo or in situ biofilms remains to be studied.

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Study design: RE, WC, JC. Performed the experiment: RE, MB, JK. Analyzed the data: RE, EZ. Wrote the paper: RE, EZ, WC, JC.

Declaration of interests

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